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TITLE: Isolation of a Breast Cancer Tumor Suppressor Gene from
Chromosome 3p

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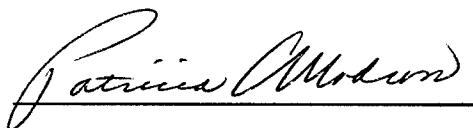
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13. ABSTRACT (Maximum 200 words) Loss of tumor suppressor genes (TSGs) represent critical molecular events in the development and progression of breast cancer. Based on loss of heterozygosity (LOH) studies as well as direct cytogenetic studies of breast tumors, one or more TSGs likely resides on the short arm of chromosome 3 (3p) and appears to be involved in nearly 50% of breast cancers. Four distinct regions within 3p [p12, p14, p21 (proximal) and p21 (distal)] undergo recurrent deletions in human carcinomas and are the most likely sites for a breast cancer TSG. In our previous Progress Reports and publications, we demonstrated recurrent homozygous deletion or rearrangement in breast cancer cell lines involving 3p14. The critical region was cloned and sequenced which led to the identification of several putative exons. We determined that 3p14 is subject to a high degree of genomic instability which is ongoing in some cases. We have also made substantial progress in evaluating other 3p regions for involvement in breast cancer, as originally proposed. This has included the 3p21.31 and 3p21.33 homozygous deletion regions as well as the interval between. In addition, we have discovered a novel patched-related gene which resides in a region of frequent amplification in breast tumors.			
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FOREWORD

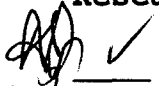
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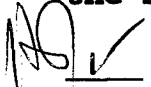
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Principal Investigator: Harry A. Drabkin, M.D.

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5. INTRODUCTION

5.A. Purpose of the Present Work

Our project concerns the identification and isolation of a breast cancer tumor suppressor gene from the short arm of chromosome 3 (3p). In the early stages of this investigation, we identified a region of homozygous deletion in a subset of breast cancer cell lines suggesting that a tumor suppressor gene would be found in the region of loss. The deletion occurred in a segment of DNA within a few hundred kilobases (1 kb = 1,000 base pairs, a measure of distance along the DNA molecule) of a chromosomal rearrangement involving chromosomes 3 and 8 which is associated with hereditary kidney carcinoma (1). (It is often the case that a tumor suppressor gene is involved in more than one type of cancer, and both kidney and breast cancers are of epithelial cell origin.) The region surrounding this breakpoint (located in band 3p14.2) was one of the possible target loci described in our original application. In our previous Progress Reports, we explained how the order of specific aims had been modified to permit detailed examination of band 3p14.2 first. This allowed us to develop necessary DNA reagents to study the target area thoroughly and to identify potential genes. The progress we described in that first report included assembly of completed sequence for over 150 kilobases of DNA and identification of a number of potential gene coding regions (exons). These data have been published (2). In the next Progress Report, we described how sequences were generated for more of the critical region and how several putative genes were identified. Importantly, we discovered that the deletion region in 3p14.2 was subject to ongoing genetic instability coincident with the common fragile site (FRA3B). We are exploring this instability phenomenon in some detail since it should provide important insights into mechanisms of genetic change which are a major feature of breast tumor cells. Our previous Progress Reports documented the efforts we were directing at three additional deletion sites which could harbor important breast cancer related genes. We described a new gene in proximal 3p21 and developed a set of contiguous PAC clones covering the most distal homozygous deletion region in 3p21. (Distal and proximal refer to directions along the chromosome, with distal meaning towards the telomere, the structure which caps the chromosome arm and proximal meaning towards the centromere.) The current Progress Report will review our new findings in all these 3p regions and we will describe a new gene currently being tested for involvement in breast cancer.

5.B Nature of the Problem

Basic concepts especially for the lay reader We realize that the scientific literature is nearly totally composed of technical terms. Throughout this report we have attempted to explain these concepts in lay terms.

The malignant potential of any tumor, including breast cancer, is a consequence of specific alterations (mutations, deletions, amplifications, etc.) in target genes that regulate the growth and biologic behavior of those cells. (Genes are segments of DNA which encode proteins; DNA is "transcribed" into RNA and RNA in turn is "translated" into protein.) Whether cells grow slowly and remain localized, or proliferate rapidly and spread to distant sites (metastasize) is a complex process involving a host of regulatory genes. For example, loss or mutation of the p53 tumor suppressor gene, located on 17p, is associated with instability of the genome (entire DNA of the cell) (3) and a worsened prognosis. This instability results in an enhanced capacity of the malignant cell to undergo DNA rearrangements leading to alterations in critical regulatory genes. Loss of normal p53 function is also associated with the cell's ability to escape death or cell cycle arrest resulting from therapeutic radiation or chemotherapy (4).

In other instances, the critical regulatory genes have yet to be identified. This is the case for genes located on 3p although some important candidates have been discovered in target regions. Scientific investigations have provided strong evidence pointing to where certain types of critical genes are likely to be located. For example, cytogenetic studies, which examine the content and

nature of chromosomes within cells, have identified certain recurrent abnormalities in cancers. Specific chromosomal segments have been found to be increased in number (amplified). This finding is expected to be associated with overexpression of a gene (because of its increased copy number). Such genes, for example, may encode growth factor receptors or may encode proteins that mediate resistance to chemotherapeutic agents. An example is provided by the MDM2 gene whose protein product inhibits the activity of p53; overexpression of MDM2 is thought to have consequences similar to mutation in p53. In contrast, cytogenetic studies have also pointed to recurrent deletions involving specific chromosomal regions. The critical genes believed to be encoded in these regions are referred to as tumor suppressor genes, the type of gene located on 3p which is the focus of our investigation.

The nature of known tumor suppressor genes is quite varied. Certain tumor suppressor genes, e.g. p16 (an inhibitor of the cyclin dependent kinases or CDKs) and the retinoblastoma gene (RB1, an inhibitor of the E2F transcription factor), control cell division by regulating the process of DNA replication. Some proposed tumor suppressor genes, such as the chromosome 18 gene DCC (Deleted in Colon Carcinoma), are located on the cell surface. DCC has been shown to encode a receptor for a netrin, a protein involved in nerve growth cone development (5). Although a mouse mutation which destroys netrin function does not generate murine cancers, this may result from differences between mice and humans (6). One of the genes we identified in the proximal 3p21.31 deletion region is Semaphorin IV which is also involved in nerve growth cone guidance. This suggests that molecules initially identified in signaling pathways associated with nerve growth cone guidance may be involved in cancer. Importantly, Yamada et al., (7) have recently demonstrated that a related semaphorin (semaphorin E) can cause non-MDR drug resistance in expressing cells. In addition, semaphorin IV has recently been implicated (8) as the gene responsible for the 3p21 driven tumor suppression observed in murine A9 cells (9). A common feature in this class of tumor suppressor genes is that their normal function is lost as part of tumor development. As a consequence, a regulatory function generally affecting growth and differentiation is also lost. The resulting cell may divide more frequently than is appropriate, giving rise to a clone or small cluster of related but abnormal cells and setting the stage for further genetic changes.

5.C. Methods to Isolate/Identify Tumor Suppressor Genes

5.C.1 Positional Cloning.

Tumor suppressor genes have been isolated by two approaches. Perhaps the most frequently used method is referred to as "positional cloning" in which the region of chromosomal loss is defined by molecular (DNA) probes and cytogenetic analysis. Because a visible chromosome deletion represents a large expanse of actual DNA, it is necessary to narrow the target region as much as possible. In one approach, this is done by using "polymorphic" probes which can distinguish between the two copies of the chromosome in question (each being inherited from one parent). In the tumor DNA, loss of one copy (by a variety of mechanisms) is referred to as "loss of heterozygosity" (abbreviated LOH). To detect LOH, a DNA probe must exist which corresponds to the target DNA in question and, importantly, this bit of DNA must exhibit a frequent, naturally occurring, variation in the population. Such variations can usually be detected experimentally and they provide a means to identify differences between the two chromosome copies in any individual tumor sample. Naturally occurring differences in DNA sequence are not uncommon although some types of DNA sequences, referred to as "microsatellites", exhibit much more variation than other types. Microsatellites consist of repeated pairs of nucleotides (usually cytosine followed by adenosine, abbreviated CA) at specific chromosomal sites with the important feature that the number of pairs is variable from person to person. Since the natural variation of markers is often a limiting factor even with microsatellite markers, this explains the general necessity to test fairly large numbers of tumor samples with different DNA probes. The goals of LOH experiments are to identify one or more target regions and to narrow such regions as much as possible prior to performing gene searches.

5.C.2 Candidate Genes.

A second approach in identifying tumor suppressor genes is through the testing of candidate genes. These candidates come from two sources; first, genes mapped within regions of recurrent deletion are all considered candidates until proven otherwise, and second, those candidates whose known function suggests they might be targets independent of chromosomal position. A number of potentially important candidate loci have been identified in 3p target regions and testing of these is ongoing. These genes include the putative coding regions within 3p14.2, the semaphorin IV and DEF-3 genes in 3p21.31 and several cDNAs identified in 3p21.33. In addition, the *patched*-related gene TRC8 is altered by a chromosomal translocation involving 3p14.2 and maps to a chromosomal region frequently amplified in breast tumors.

5.C.3 Identification of Homozygous Deletions Facilitates Positional Cloning of Tumor Suppressor Genes

LOH studies can lead to the identification of homozygous deletions in tumors, an extremely important finding which can greatly facilitate the precise positioning of tumor suppressor genes. Furthermore, homozygous deletions provide one of the best ways to choose particular candidate genes from a larger set, based upon their location within the minimal deletion defined experimentally. These often rare events thus provide a powerful adjunct to LOH and candidate gene studies.

A homozygous deletion means that both copies of a chromosome have undergone loss for a particular segment of DNA (see diagram below).

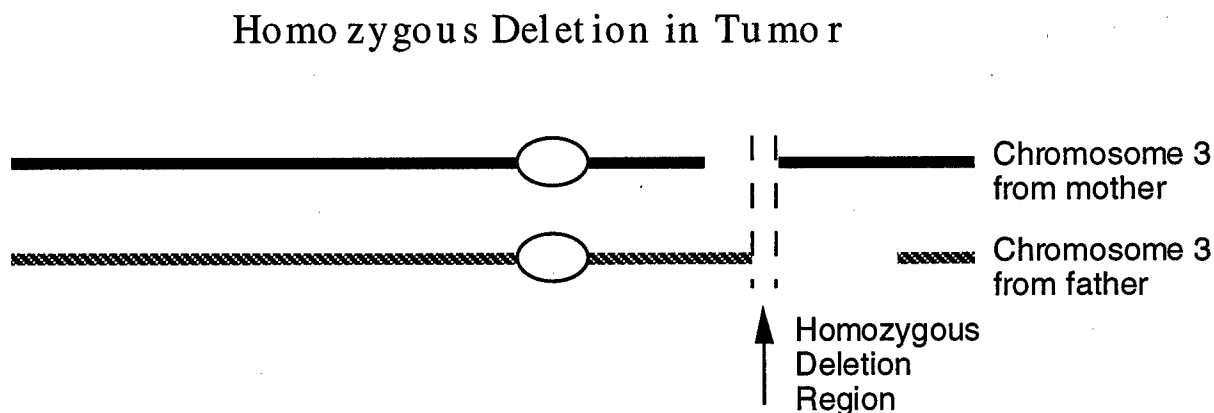


Figure 1. Diagram of chromosome 3 homologues with overlapping deletions in a tumor.

Such deletions represent one of several mechanisms that can lead to the complete loss of a tumor suppressor gene. While tumors frequently lose one tumor suppressor gene copy by undergoing large chromosomal deletions, the remaining copy is usually rendered non-functional by other genetic mechanisms. These can include point mutations and gene silencing (caused by hypermethylation) as well as a second deletion leaving a complete gap in the genetic material.

5.C.4 Additional Background Information.

In this section, we present a summary of data from the literature regarding genetic deletions of chromosome 3 in breast cancer. These selected studies relate to all the results we have obtained to date as well as our continuing efforts to implicate 3p-encoded genes in breast cancer. We have

placed these comments here for the convenience of the reader, since the concepts of loss of heterozygosity (LOH) and homozygous deletions have been introduced earlier.

Sato et al. (10) examined 120 breast cancers for loss of heterozygosity (LOH) using a series of 3p polymorphic loci. Nearly 50% of the informative tumors (56/120) demonstrated LOH involving 3p. In this study, the 3p region undergoing the greatest loss was 3p14.2-p13 which should include the homozygous deletion region we have identified. This study has been one of the largest in terms of the number of tumors examined and 3p-derived probes tested. One significant limitation was that the type of probe used did not detect sufficient polymorphisms to allow the limits of the 3p target region to be defined more precisely. A cytogenetic study (chromosome analysis by direct microscopic visualization) by Pandis et al. (11) identified 3p deletions in 5 of 41 breast carcinomas. These deletions appeared nearly identical and involved the 3p13(p14) region implicated above using LOH analysis. Intriguingly, in 3 of the 5 cases studied by Pandis et al., (11) the 3p deletion was seen as the only recognizable cytogenetic abnormality. This suggests that 3p deletion may be an early event in a subgroup of breast cancers. Another study using LOH analysis demonstrated that 3p loss was most frequently seen in familial breast cancers (12). The highest frequency of loss (68%) was with a probe (D3S1217) also located in the 3p14 region. These observations are significant since both BRCA1 and BRCA2 appear to be involved in recombination and DNA repair (13,14). Our previous results have suggested that 3p14 deletions result from the loss of an inherently unstable segment of DNA which coincides with the 3p14 common fragile site (2). These deletions may be caused by recombinational errors, which would render this segment particularly sensitive to loss in a background of BRCA1 or 2 mutations. Taken together, there is considerable evidence from multiple investigators using different techniques which supports the frequent alteration of 3p14 during breast cancer development.

Additional data from LOH and cytogenetics studies implicate other 3p regions that may harbor genes important in breast cancer. Chen et al (15) found LOH within 3p13-14, 3p21-22 and 3p24-26 along with one case of homozygous deletion in 3p13 likely corresponding to our FRA3B associated homozygous deletions. All three of these target sites have been previously implicated in other carcinomas, particularly of lung. Hainsworth et al., (16) found frequent chromosomal breakpoints at 3p21 which can be indicative of gene disruption or deletion. In a study by Ali et al, (17), the shortest region of common loss was between the markers D3F15S2 and RAF1, spanning the chromosomal region 3p21-p25 and a similar study by Devilee et al (18) found the p14 to p21 region commonly deleted. Several other studies corroborate these findings having observed interstitial 3p deletions (19) or other structural changes affecting this chromosome (20). Intriguingly, Buchagen et al., (21) identified one apparent rearrangement and one homozygous deletion for the probe D3S2, known to map within proximal 3p21.1 (22) very close to the semaphorin IV deletion region (23). These studies all implicate multiple target regions on 3p which appear to be very similar to regions identified from studies on carcinomas derived from other tissues. Based on these studies, we have focused efforts on three homozygous deletion regions. We have demonstrated that 3p14 is inherently unstable in tumor cells with p53 mutations, and appears to be particularly susceptible to genetic losses in this situation. We believe that instability is the primary event affecting 3p14, although we have not yet ruled out the possibility that a tumor suppressor function is also lost. These experiments are ongoing. We are exploring ways of influencing the rate of new deletions by introducing genes which are known to activate recombination. If deletion rate can be affected, the results should provide important insights into the mechanism(s) for this common alteration. Furthermore, it may be possible to use the status of 3p14 as a biomarker for diagnostic or prognostic tests. We are also carefully examining two candidate genes in proximal 3p21 and have completed a search for homozygous deletions over an estimated 750 kb of the distal deletion region in 3p21.

5.D. Review of Specific Aims

5.D.1. Our original Specific Aims were designed to:

1. Define the regions of 3p undergoing LOH in breast cancer.
2. Test known 3p candidate genes for mutations in breast tumors.
3. Isolate additional candidate tumor suppressor genes from regions we define.
4. Characterize the product(s) of these genes and assess their involvement in breast cancer.

In our first Progress Report, we described how these aims were modified early on to reflect the substantial effort being focused at that time on the recurrent homozygous deletion region involving 3p14. In subsequent Progress Reports, we noted that many of these modified goals had been achieved and we provided a detailed account of the results. A manuscript describing our cloning of FRA3B, discovery of homozygous deletions in the region and sequence analysis was published (2). We subsequently pursued several other sites where potential breast cancer tumor suppressor genes might be found, following the original strategy and aims of our proposal. We have continued to make significant progress on these aims during the past year. Below, we briefly summarize our past progress, since the details of these studies were provided in previous reports and in publications. We then describe our accomplishments in the past year in detail. These include testing breast cancer lines for deletions in the distal 3p21 region and investigating the function and potential role for genes in the proximal 3p21 region. In addition, our efforts to understand the 3p14 region have led to the discovery of a new *patched*-related gene, TRC8 (24), which is altered in a hereditary cancer. Although TRC8 does not map to 3p, it is located in a region of frequent amplification in breast tumors (8q24).

6. BODY

6.A. Summary of previous findings.

6.A.1. Examination of breast cancer cell line DNAs for homozygous deletions in 3p14.

Southern blot hybridizations with probes from 3p14 detected homozygous deletions or rearrangements in DNAs from breast cancer cell lines (2). Of the 13 breast carcinoma cell lines tested, one contained a discontinuous deletion while two were apparently rearranged. We developed a ~300 kb cosmid/lambda (DNA clone) contig in the region of the homozygous deletions (2). From sequence and hybridization data, we showed that the cloned region corresponds to the most inducible common fragile site in the genome, FRA3B (25). Fragile sites are DNA regions which are unstable. In a few cases of rare fragile sites, their nature has been elucidated at the DNA sequence level and appears to be due, at least in part, to an expanded triplet repeat which may interfere with normal DNA replication. Our results clearly demonstrated that FRA3B represents a region rather than a single site and does not contain an obvious triplet repeat. Where we have accurately defined the boundaries for the carcinoma-associated deletions, one or both are contained within FRA3B, showing that these deletions overlap the fragile site.

During our studies, Ohta et al. (26) identified the FHIT gene from 3p14, reporting frequent abnormalities in RT-PCR products from carcinoma cell lines derived from colorectal and lung tumors. (In RT-PCR experiments, RNA is isolated and reverse transcribed into cDNA. PCR primers are then used to greatly amplify this product using the polymerase chain reaction which can then be examined for its correct size or its DNA sequence can be determined.) These observations were subsequently extended to breast carcinomas (27-29). In our previous Progress Reports, we outlined multiple reasons for skepticism about these results. Our own studies have led us to conclude that 3p14 is inherently unstable, particularly in tumors with p53 mutations (2). This instability appears to be the primary driving force for deletions affecting 3p14. All of the data

regarding the FHIT gene can be readily understood as the result of a bystander effect in which FHIT is damaged as a consequence of deletions within an unstable region. Whether or not these deletions confer a selective advantage to tumor cells is to date an unanswered question.

6.A.2 DNA Sequencing and Analysis

We undertook DNA sequencing studies as a means to identify genes and to understand the instability of the region (2). DNA sequencing has been completed and deposited in the public databases for approximately 180 kb and our analysis of the sequence features has been published for a contiguous stretch of 110 kb (2). (The DNA sequence analysis involves computer algorithms to identify all repetitive DNA sequences, identities and similarities to known genes contained in various databases, predicted gene segments and various other structural/compositional features.) We found the region to be very high in A-T base pair content with frequent LINE and MER repetitive elements, and conversely low in Alu repetitive elements and confirmed genes. In contrast to the reported rare folate-sensitive fragile sites, which are associated with expanded CGG repeats (30,31), FRA3B does not contain an expanded triplet repeat or methylated CpG-island. More recently, Inoue et al., (32) have sequenced 207 kb of this region which partially overlapped our data and extended the sequence by 130 kb in the telomeric direction. These additional data show the region continues to be AT rich and lacks a triplet repeat. Our sequence data identified a number of putative exonic sequences which we have investigated; the results are described briefly below.

6.A.3 Additional genes in FRA3B and 3p14?

As we described in detail in our previous Progress Report, sequence data revealed a number of potential gene encoding regions within FRA3B in addition to FHIT. Many exons were predicted by GRAIL2 and GeneMark exon prediction programs (33), but nearly all occurred within repetitive elements of the LINE and MER families. Some showed identity with an EST from the dbEST sequence database, but these were discarded as candidate genes because they appeared to be primed from a genomic poly-A tract and were co-linear with genomic DNA. (ESTs are short DNA sequences obtained from the ends of cDNA clones. While most cDNA clones represent parts of genes, there are some which are derived from incompletely spliced RNA and others which may represent DNA contamination in the library.) Of five highly predicted exons which remained, none have shown similarity to known genes. Several exons with lower probability scores occurred in regions which were identified by other experimental evidence. Thus, this portion of the deletion region has the potential to encode more genes than FHIT.

We previously described results on several putative exons including λ 58, GB and HRCA1. Despite the evidence from RT-PCR experiments suggesting expression of these three putative genes, all were found to be negative on Northern blots. We now believe that these putative exons were expressed as read-through transcripts. The large amount of sequence data now available from our efforts and from Inoue et al., (32) provides a much more in depth analysis of the region. At this time, there appears to be no compelling evidence that any additional genes are present in this region. We also undertook a series of functional tests to identify potential tumor suppressor genes within YAC clones (yeast artificial chromosomes) containing human DNA from 3p14. We introduced a selectable marker (by retrofitting) into YAC 74B2g and transfected the intact YAC into murine A9 cells. Initial testing of the tumorigenic potential of A9 parental and 74B2g transfected cells in *nude* mice suggested that the 74B2g transfectant was highly suppressed in its ability to form tumors. These experiments have now been repeated and no differences were observed. These observations illustrate the difficulties in performing such analyses. It is likely that the original evidence for tumor suppression was due to clonal variation.

The results which we have summarized above suggested that the only gene within the 3p14/FRA3B region was FHIT. However, this left unanswered the question of what the 3;8

translocation was doing to induce hereditary cancer. We now know the answer to this question involves the TRC8 gene, related to the sonic hedgehog receptor, patched, which we describe in more detail below.

6.A.4. TRC8.

The hereditary 3;8 translocation associated with 3p14 provided one of the strongest reasons for believing that this band contained a tumor suppressor gene. It had been assumed by many investigators that the translocation interrupted a tumor suppressor gene which was also deleted in many sporadic carcinomas including breast tumors. Although FHIT appeared to meet these expectations when first discovered, a substantial body of evidence now exists refuting this. As a result, we explored the alternative possibility that this translocation involved a gene on chromosome 8. The recent observation by Geurts et al., (34) that FHIT was fused to the HMGI(C) gene in a benign parotid adenoma suggested that a similar fusion might result from the (3;8) translocation. To further our understanding of the genetic events which can affect 3p14, we undertook RACE experiments to identify any such gene fusions. We discovered that FHIT is indeed fused to a novel chromosome 8 gene, which we named TRC8 (24), and that this gene showed significant similarity to *patched* (PTCH), the receptor for the *sonic hedgehog* (SHH) signaling molecule. This is of major significance for several reasons. Mutations in PTCH are responsible for the basal cell nevus syndrome, a hereditary form of skin cancer, and are frequently found in sporadic basal cell carcinomas as well as meduloblastomas. Thus TRC8 is related to a known tumor suppressor gene and may function in an analogous fashion. However it is important to understand that we do not yet know if TRC8 will act as a recessive tumor suppressor or as a dominant oncogene. This region has been demonstrated by Wigler et al., (35) to harbor several independent amplification domains in breast tumors. Thus it is possible that TRC8 is affected by one of these amplification domains and we intend to explore this possibility. We have established a collaboration with Dr. Fujio Kasumi at the Japanese Foundation Cancer Institute in Tokyo who has provided matched tumor/normal DNA samples from breast tumors. These samples will be analyzed for TRC8 copy number and where possible, for levels of TRC8 expression. Such data should help establish whether or not TRC8 is a target for amplification in breast cancers.

6.A.5. Summary and Significance of 3p14 Findings

The identification of a DNA segment which undergoes recurrent homozygous deletion and rearrangement, as we have observed for this region of 3p14, initially suggested that it encoded a tumor suppressor gene. However, at the present time our data do not support FHIT as the 3p14.2 tumor suppressor. We proposed an alternative hypothesis; that genomic instability *per se* may lead to the observed deletions. While there are putative exons, there is no further support that these represent functional genes. These sequences have no matches in databases and we have observed no evidence for expression by Northern blots. To the best of our knowledge, this region undergoes loss as a result of genomic instability and not through selection against a tumor suppressor gene (2). We were the first to propose this alternative hypothesis for the origin of 3p14 deletions. It is based upon our detailed sequence analysis, gene searching, deletion analysis and functional assays, much of which has been supported under the auspices of this grant. We find it very interesting however that there are marked differences in the rates of *de novo* deletions involving this region in different tumor cells. These differences are not explained by p53 mutations and suggest additional alterations which influence genomic instability in this region. Such mechanisms would have the capacity to effect the development and progression of breast cancer and are discussed below.

(UNPUBLISHED DATA)

6.B. Summary of unpublished data.

6.B.1 Ongoing instability of 3p14.

In our last Progress Report, we described experiments to determine if 3p14 and FRA3B sequences were inherently unstable in carcinoma cell lines. This possibility was suggested by the often discontinuous deletions we observed in carcinomas, including the breast tumor MDA231. Ongoing instability of 3p14 was considered to be the most likely explanation for this result. We reasoned that if the region was inherently unstable, then carcinoma cell lines which still retained much of the region might be progressively deleting more of FRA3B and these events could be measured. Our strategy was to subclone two lines with known deletions (Fig. 2) and to analyze their DNA for alterations within FRA3B using PCR and Southern blots. We previously reported that 11 of 25 CC19 subclones (44%) had new deletions, no two of which were identical. We have now extended this analysis for a total of 33 sub-clones and find that 16 contain new deletions (48%). These results could be explained by ongoing instability or by the sub-cloning of variants which were already present in the population. To distinguish between these possibilities, we performed a second sub-cloning experiment using a previous sub-clone of CC19 which showed no evidence for new deletion (CC19 sub-clone-7). We reasoned that if instability was truly ongoing, then a sub-clone without new deletions should show evidence for instability after a period of growth followed by sub-cloning once again. We grew CC19 sub-clone-7 for 10 passages, re-subcloned it and analyzed 64 sub-clones finding two (3%) with definitive evidence for new deletions. This low rate suggested that deletions in the original sub-clones had accumulated over time, while the presence of new deletions in CC19 sub-clone-7 derived cells confirmed that deletions were ongoing. In contrast, the cell line MDA231 has shown no evidence for new deletions. Thus we have identified a major difference between cell lines, both of which contain p53 alterations and 3p14 deletions. In one case the deletions are ongoing at a high rate while in the second the deletion appears stable. The nature of this fundamental difference is at present unknown.

We are extending these results further by examining the effects of activated RAS and MAP kinase kinase (MKK) pathways on this deletion rate. Activated RAS has been shown to induce chromosome breaks and other aberrations in NIH 3T3 cells with p53 mutations. In collaboration with Dr. Natalie Ahn (Univ. of Colorado, Boulder) we have introduced a mutant RAS expression construct into CC19 cells. The RAS mutation replaces the histidine at condon 61 with a leucine, rendering the protein GTPase deficient and constitutively active. RAS H61L has been incorporated into the pZIP mammalian expression vector and is expressed under control of the CMV promoter. RAS H61L transfectants of CC19 and control parental cells have now both been subcloned (43 parental and 38 RAS transfectants subclones, respectively). We are in the process of testing DNA from these cells for the presence of new 3p14 deletions and to determine if activated RAS alters deletion rates. However, RAS signals feed into multiple and diverse downstream pathways, only one or a few of which may lead to genetic instability. With our CC19/FRA3B deletion system we can explore signal transduction pathways for their influence on deletion mechanisms. The first components to be tested will include the MAP kinase kinases MKK1 and MKK2. Dr. Ahn has developed constitutively active mutations for both MKKs which remove N-terminal amino acids and substitute key residues in the active site with acidic amino acids. The substitutions mimic the effects of phosphorylation in the active site and render the mutant's kinase domains constitutively active. The primary downstream targets for MKKs are ERK1 and 2, which when activated by phosphorylation will modify a wide array of target proteins including transcription factors, structural proteins and other kinases. When transfected into CC19, we will be able to determine if this specific downstream pathway leads to increased genomic instability. Similar approaches can be used to assay the influence of other downstream components of the RAS pathway.

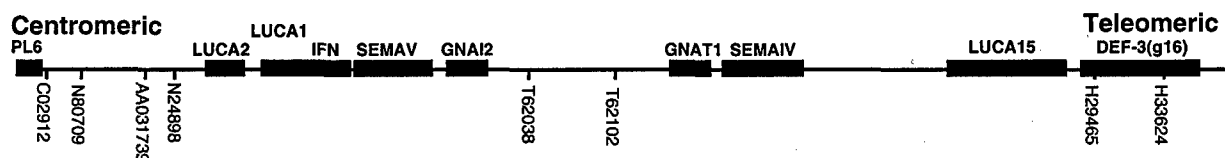
6.B.2. Summary and Significance of 3p14 instability.

Overall these results show that 3p14 is inherently unstable in at least a subset of tumors. Our efforts to understand the specific mechanisms leading to instability of FRA3B should provide significant insights into this overall process which is such a key event in development of breast cancers. Furthermore, this understanding could lead to improved tests for detecting such changes in the early stages of carcinogenesis.

6.C. Involvement of 3p21.31 in Breast Carcinoma.

6.C.1. Known and Predicted Genes in 3p21.31.

In our last Progress Report, we reviewed the evidence that 3p21 harbors one or more potential tumor suppressor genes. First, the region has the highest frequency of LOH in many carcinomas and has been the target of homozygous deletions in cell lines and uncultured tumors. Importantly, the involvement of this region has been repeatedly suggested by LOH studies in breast cancer. Secondly, functional studies by Killary et al., (9) have shown that DNA segments from this region will suppress tumorigenicity of the mouse fibrosarcoma cell line, A9. We also reviewed knowledge about genes found within the common deletion region. Clone contigs and continuous DNA sequence data are available for this entire region and over 10 genes have been identified. The major genes in this interval are shown in Fig. 3 below.



Two of these genes, Semaphorin IV and DEF-3, were isolated by our efforts (23,36) and we have made substantial progress in understanding their structure and functions (section 6.C.3 and 6.C.4, below).

6.C.2. Analysis by PCR for 3p21.31 Homozygous Deletions in Breast Carcinomas.

In our last report, we described how we had completed a detailed search for homozygous deletions in breast cancer lines using the polymerase chain reaction (PCR). PCR analyses were used to assess these lines for homozygous deletions in the 3p21.31 area corresponding to the map in Fig. 3. PCR results were obtained for 12 cell lines with 11 markers corresponding to each gene in the region. This analysis found no evidence of homozygous deletions with this set of cell lines and markers. However, another group has identified a homozygous deletion in a breast cancer cell line involving this region (37).

6.C.3 Investigation of Semaphorin IV.

The rationale for studying this region derived from three overlapping homozygous deletions in small cell carcinomas, one of which we identified. Furthermore, any clear tumor suppressor gene is likely to be involved in more than one type of carcinoma. Within this deletion region, we as well as two other groups identified the gene H.Semaphorin IV (23,38,39), a member of the semaphorin/collapsin family. While the function of this gene family was initially associated with the migration of nerve growth cones, the broad expression patterns, gene knock-out studies and

the recent finding that semaphorin E could mediate multidrug resistance (7) argues that these genes are extremely important in non-neural development.

We described the H.Semaphorin IV gene in detail in a previous Progress Report. Semaphorin E, related to Semaphorin IV, has recently been shown to be responsible for at least some drug - resistant phenotypes encountered in tumor cells (7). Yamada et al., (7) selected for cDNAs which were capable of conferring resistance to cis-platinum in Cos-7 cells. The primary clone recovered was semaphorin E and transfection of this clone could confer resistance to other cell lines.

The region of 3p21.3 including semaphorin IV was shown by Killary et al., (9) to confer tumor suppression when introduced into murine A9 fibrosarcoma cells. In subsequent studies using individual P1 clones, semaphorin IV was implicated but the data were inconsistent (40). Very recent studies from the Naylor lab (8) have provided additional evidence that semaphorin IV may mediate the suppression of A9 tumorigenesis. Whereas parental A9 cells undergo apoptosis in response to chemotherapeutic drugs, semaphorin IV expressing clones do not and are instead growth arrested. It is also very interesting that the receptors for semaphorin IV include neuropilin 1 and 2, and recent data indicate that the angiogenic factor, VEGF₁₆₅, also binds the neuropilins. Thus there is the intriguing possibility of complex interactions between the secreted semaphorin and VEGF₁₆₅, in the regulation of angiogenesis and perhaps cell migration. Semaphorin IV may indeed be a critical gene in breast tumors, despite the absence of clear deletions in these tumors.

Our studies on the semaphorin molecule have included the development of polyclonal antibodies specific for semaphorin IV. Because of the high degree of conservation among the family members, this was not a trivial task. While these antibodies (directed against a unique peptide) appear to work well for immunohistochemistry, they do work on Western blots. Our current studies involve the intracellular localization of semaphorin IV and its possible alteration in tumors. We think this is a very fruitful area for investigation. We have also generated mammalian expression constructs for semaphorin IV and introduced these into mouse A9 cells. Unlike the results from the Naylor lab (8), we have observed no difference in tumorigenicity. However, while expression of the recombinant gene was clearly evident at the RNA level, we have been unable to clearly demonstrate the presence of recombinant semaphorin IV protein. The difficulties include the following; 1) the mouse and human genes are very identical, 2) semaphorin IV is a secreted molecule with a signal sequence at its amino terminus such that epitope tags cannot be placed in this region, 3) the carboxy terminus of the semaphorins have been reported to undergo furin mediated cleavage making this site problematic for an epitope tag. Analysis of the transfected A9 cells using our polyclonal antibody indicates that a small subgroup express the human protein at high levels. We think this is due to the variable copy number of the episomal vector used for the construct. To pursue this in more detail, we have developed a non-episomal construct and have entered into a collaboration with Dr. John Minna (UT Southwestern, Dallas) to exchange expression constructs and to clarify the issue of tumor suppression in A9 cells. Of note, the deletion in the breast cancer cell line, identified by the Minna lab, does not contain semaphorin IV. This is in direct contrast to the functional studies performed by the Naylor lab. This 3p21.3 deletion region has been totally sequenced by the Washington Univ. and Sanger sequencing Centers. While the region is gene rich, no tumor specific mutations have yet been reported. There are several gene duplications in the region which may give rise to a higher frequency of deletions or aberrations. This makes the functional studies of this region important for the identification of the putative tumor suppressor gene in this region.

6.C.4 Investigation of DEF-3(NY-LU-12/g16)

We noted that each of the initial three homozygous deletions involving 3p21.31 appeared to be clustered at their telomeric boundaries. Therefore, we looked for a gene which might be commonly interrupted by these alterations. DEF-3 is a novel sequence which was isolated originally by us as a partially processed cDNA (L1-204) containing a single, very small exon (23). We have now

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cloned the full length cDNA from both human and mouse and found that the predicted protein has significant homologies to RNA binding proteins (Fig. 4, below) with multiple domains for RNA binding and protein-protein interactions.

hDEF-3 (g16/NY-LU-12).



Fig. 4. Domains of hDEF-3. RNA recognition motifs (RRM), octamer repeat (8mer), coiled coil (CC), zinc finger (ZnF) and nuclear localization signal (NLS) are indicated.

RNA binding proteins are involved in a diversity of biological functions affecting RNA processing, half-lives and expressibility. Interestingly, antibodies against RNA binding proteins have been identified in some breast cancer patients with the POMA syndrome resulting from antibodies directed against the RNA binding protein Nova-1 (41). Gure et al., (42) have independently identified antibodies against DEF-3/NY-LU-12 in some patients with lung cancer. These observations raise the possibility that antibodies against DEF-3 could be related to certain deletions in 3p21. Our efforts on this gene have led us to define a new family of RNA binding proteins and to demonstrate that DEF-3 and an adjacent, co-deleted, family member (LUCA15) bind poly (G) homopolymers *in vitro* (Fig. 5). Moreover, the RNA recognition domains of DEF-3 and LUCA15 are non-reactive with antibodies from patients with the Hu syndrome. Anti-Hu sera were tested because the RRM of DEF-3 and LUCA15 are similar to those from the Hu proteins. We have developed antibodies against the RRM domains of DEF-3 which are undergoing characterization and which would permit immunologic detection of deletions in direct tumor materials and facilitate analysis of this region.

6.C.5. Summary and Significance of 3p21.3 Findings

The 3p21.3 deletion region appears to be a promising area for the identification of a tumor suppressor gene. One of the best candidates to date is the semaphorin IV gene, cloned in our laboratory. Our experiments to date address primarily aims 2, 3 and 4 for this region.

6.D. Involvement of 3p21.33 Region in Breast Carcinoma

6.D.1. Screening Breast Tumors for Deletions in the 3p21.33 Deletion Region.

Chromosomal band 3p21 harbors a second homozygous deletion region, originally described by Yamakawa et al., (43). They went on to show that the minimal deletion spanned approximately 800 kb and was covered by YAC 936C1. We demonstrated that the deletion target was separated from the semaphorin IV deletion site by 10 to 15 megabases of DNA (23). In the course of mapping this region more thoroughly, we discovered that one of our markers, Mbo16E2, was present in this YAC (23). In our last two Progress Reports, we described how the YAC 936C1 was found to be chimeric and thus useless for developing additional markers in this interval. We proceeded to generate an independent clone contig using P1 artificial chromosomes (PACs) which covers at least 750 kb (Fig. 6, below).

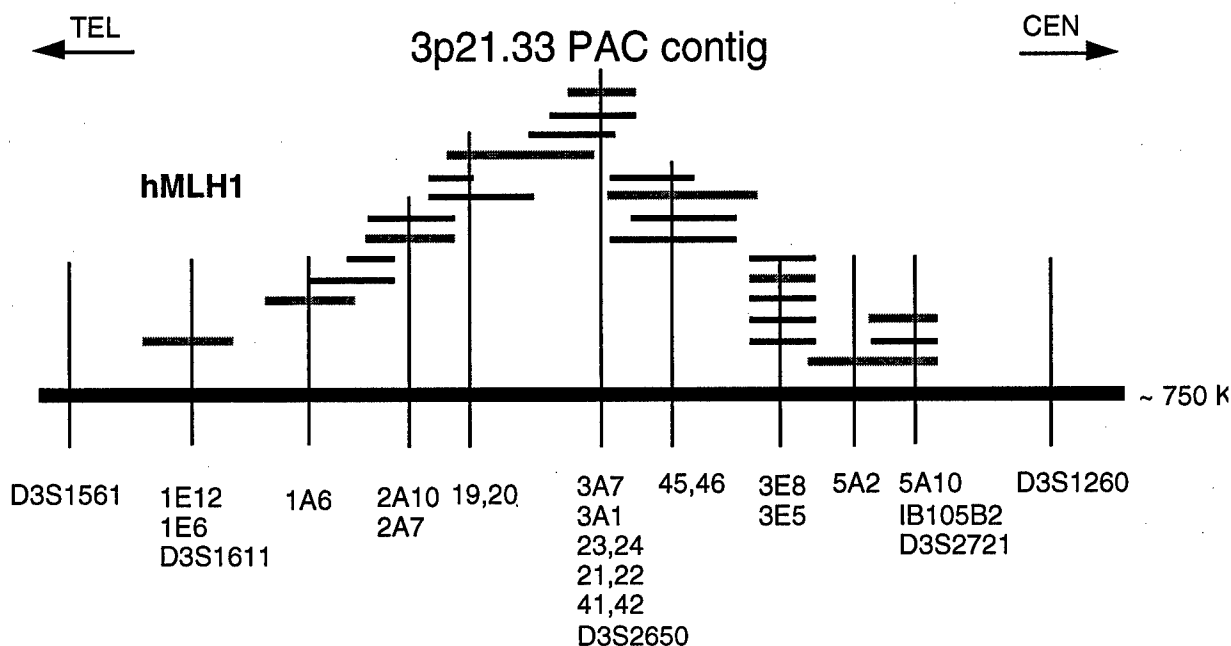


Fig. 6. PAC clone contig covering the 3p21.33 deletion region. Clones are symbolized by short horizontal lines, the shaded lines represent PACs which were sub-cloned and used to generate markers. A sub-set of the markers utilized for screening breast tumors are listed below the chromosome (thick solid line) with their approximate location. The contig is estimated to span 600 to 750 kb.

We have prepared sub-clone libraries from many of these PACs, sequenced selected clones and developed PCR primer pairs suitable for deletion analysis. Twenty six STS markers from this region have been used to screen 12 breast tumor cell lines. These markers included specific primer pairs for two genes; the mismatch repair gene hMLH1 and Integrin- α_{RLC} . The screen showed that none of these breast tumors harbored deletions of this region. However, the number of tumors examined is small. This will be expanded by collaborative studies with other investigators and these reagents will prove invaluable for the analysis of this region.

6.D.2. Significance

This region was one of the initial target deletions proposed in our application. We have now developed the reagents necessary to thoroughly examine the interval for alterations in breast tumors. These studies address aims 1 through 3.

7. CONCLUSIONS

In each of the previous sections comprising the Body of this report, we have provided a summary of our important results and their significance. Our studies continue to explore several distinct homozygous deletion regions on 3p. For 3p14, we have previously provided strong evidence that the postulated tumor suppressor gene, FHIT, is not the target of these alterations. We are exploring the observation of genomic instability, attempting to define the signalling pathways which influence it.

Our studies for two additional homozygous deletion regions (proximal 3p21.3 and distal 3p21.3) have entered new phases. Two candidate genes are being studied in the proximal 3p21.3 deletion region; expression constructs and antibodies have been generated for both. Our initial results have confirmed the RNA binding properties of DEF-3. We plan on investigating the

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expression of semaphorin IV in breast tissues and tumors, especially in light of the exciting possibility that this gene, which we originally cloned, may be the long sought after 3p21 tumor suppressor. For the distal 3p21.3 deletion region, we have begun a thorough investigation for deletions, but this must be extended to more samples.

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9. APPENDIX.

Figure Legends:

Figure 2. Map of clone contig and deletions in 3p14.2

A. Schematic showing position of the homozygous deletion region with respect to YACs 850A6, 65E7 and 74B2. Positions of *Mlu*I and selected *Xho*I restriction sites are indicated. *Xho*I sites were not determined for YAC 850A6. Novel genes λ 58, GB and HRCA1 as well as the FHIT gene are indicated in approximate position. For FHIT, the approximate positions of exons (numbered 1 through 10) are shown. HRCA1 is within FHIT intron 3, GB is in FHIT intron 4 and λ 58 is within FHIT intron 5.

B. Cosmid and lambda clone contig spanning the homozygous deletion region within 3p14. Solid horizontal lines represent cosmid (c) and lambda (λ) inserts, as indicated, along with cleavage sites for *Eco*RI (short vertical bars), *Xho*I (X) and *Sal*I (S) which were mapped within the central 170 kb. The *Mlu*I site corresponding to the site in YAC 74B2 is present in c55D12 and c84F12, although we have not determined its precise location. Positions of selected markers, breakpoints and integration sites for HPV-16 and pSV2neo are indicated across the top. The shaded bar indicates the extent of genomic sequencing accomplished so far; the scale bar indicates kilobases. Parts B and C of this figure are drawn to the same scale and positions correspond exactly between the two.

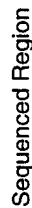
C. Homozygous deletions in tumors and normal genomic DNAs. Horizontal lines denote the extent of deletion in the indicated cell lines. Parallel lines at the ends of each deletion indicate where precise boundaries were not determined. HeLa and MDA231 contain discontinuous deletions indicated by interrupted lines.

Figure 5. Results of ^{35}S labelled protein binding to RNA homopolymers.

Lanes 1 and 2 contain approximately 10% of the in vitro transcription/translation (TnT) reaction products (^{35}S labeled) from HuD (a positive control) and DEF-3, respectively. Lanes 3-8 represent HuD binding to the indicated RNA homopolymers or corresponding matrices. The DEF-3 results are in lanes 9-14 and 15-19. HuD binds preferentially to homopolymeric(A) RNA (lane 6). DEF-3 binds homopolymeric(G) RNA (lanes 14 and 16) which is inhibited by high salt (lane 17). The results of UV cross-linking (lane 19) demonstrates that a protein - RNA interaction is necessary for binding to occur. The LUCA15 results are shown in lanes 20-26 with preferential binding to homopolymeric(G) RNA. Negative control reactions with firefly luciferase are in lanes 27-33.

22

homozygous deletion region containing FRA3B



Non-tumor

Cervix

Colon

Lung

Breast

MS751

CC19

C33A

SiHa

Caski

HeLa

HT29

COLO205

SW480

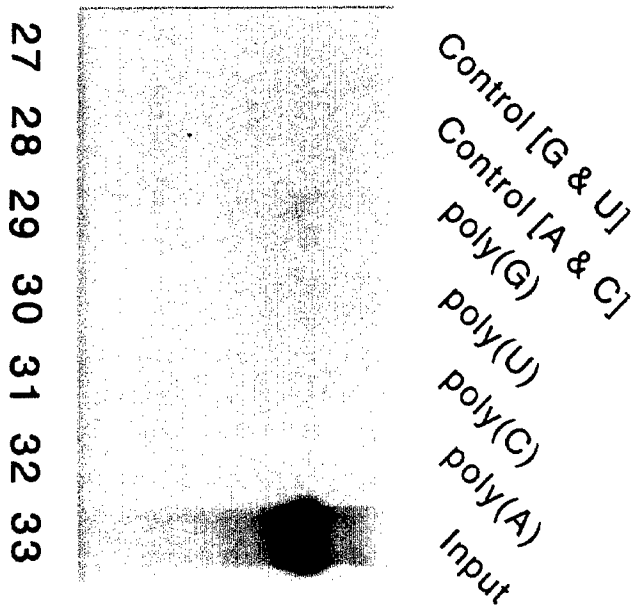
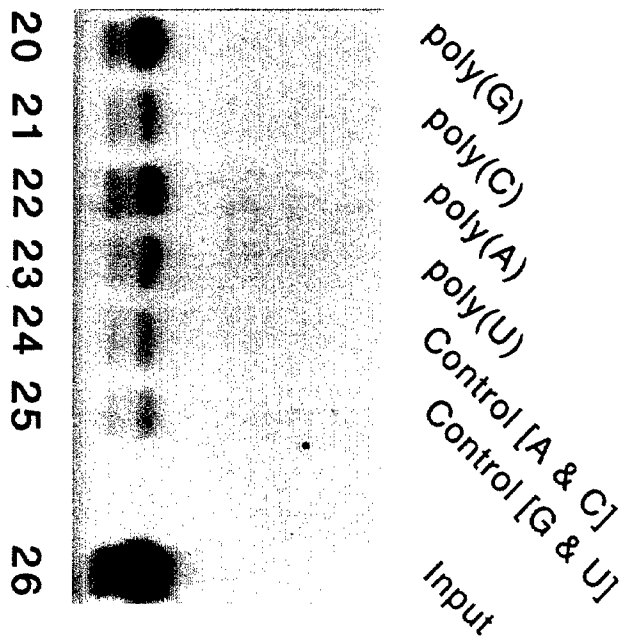
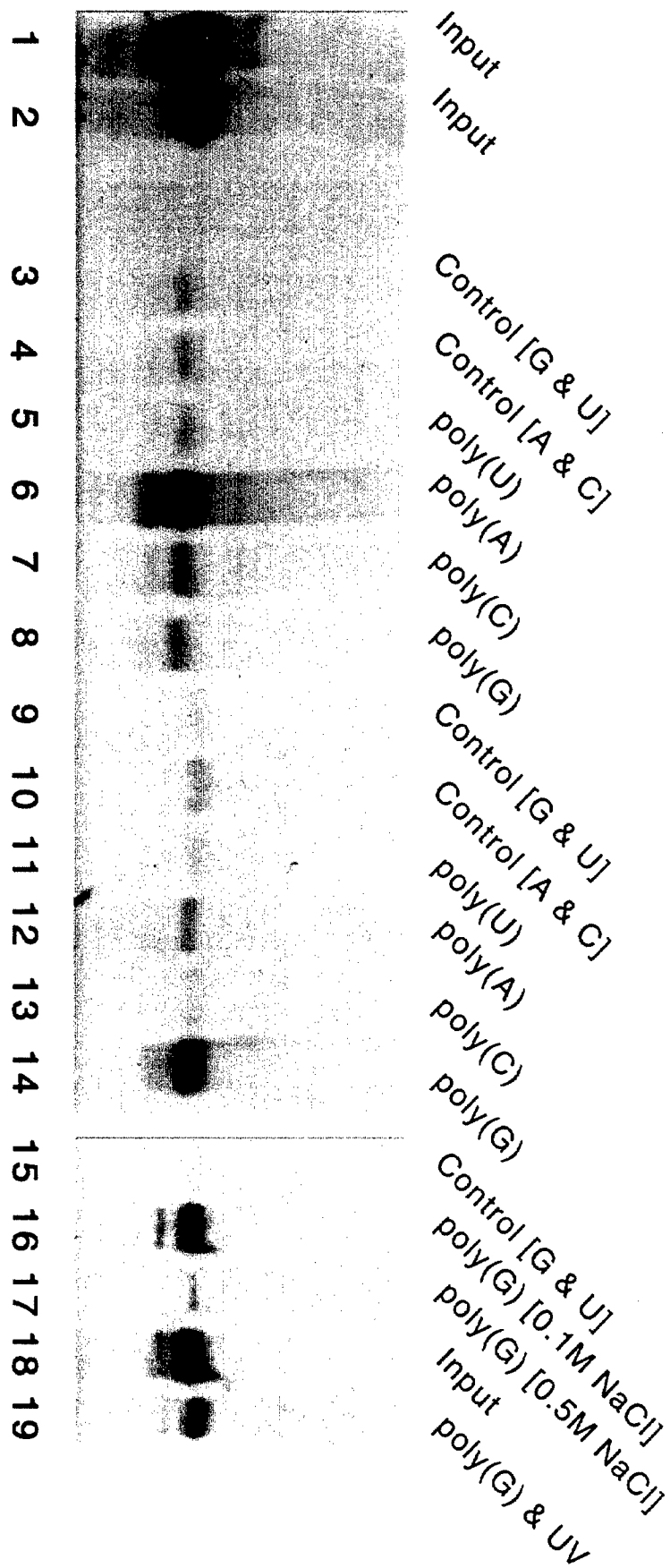
NCI-H1648

NCI-H892

NCI-H460

MDA231

MDA231





DEPARTMENT OF THE ARMY

US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

5 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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